



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US91/07654 <b>(22) International Filing Date:</b> 18 October 1991 (18.10.91)  <b>(30) Priority data:</b> 600,024                      18 October 1990 (18.10.90)      US  <b>(71) Applicant:</b> CREATIVE BIOMOLECULES, INC. [US/ US]; 35 South Street, Hopkinton, MA 01748 (US).  <b>(72) Inventors:</b> OZKAYNAK, Engin ; 44 Purdue Drive, Mil- ford, MA 01757 (US). OPPERMANN, Hermann ; 25 Summer Hill Road, Medway, MA 02053 (US). KUBER- ASAMPATH, Thangavel ; 6 Spring Street, Medway, MA 02053 (US). RUEGER, David, C. ; 150 Edgemere Road, Apt. 4, West Roxbury, MA 02132 (US).		<b>(74) Agent:</b> PITCHER, Edmund, R.; Testa, Hurwitz & Thi- beault, Exchange Place, 53 State Street, Boston, MA 02109-2809 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), NL (European patent), SE (European pa- tent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> OSTEOGENIC PROTEIN  <b>(57) Abstract</b>  Disclosed are 1) the cDNA and amino acid sequence for a murine polypeptide chain, mOP-1, useful in dimeric osteogenic proteins, 2) methods of producing osteogenic proteins using recombinant technology, 3) methods of producing osteogenic devices comprising mOP-1 dispersed in xenogenic bone matrices, and 4) use of the osteogenic devices to mimic the natural course of endochondral bone formation in mammals.		

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## Osteogenic Protein

### Background of the Invention

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This invention relates to a novel polypeptide chain and to osteogenic proteins comprising this polypeptide chain which are capable of inducing osteogenesis in mammals, to a gene encoding the polypeptide chain, to  
10 methods for its production using recombinant DNA techniques, and to bone and cartilage repair procedures using the osteogenic proteins.

Mammalian bone tissue is known to contain one or  
15 more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the  
20 literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

The developmental cascade of bone differentiation  
25 consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

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Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone

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dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo. Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

The potential utility of these proteins has been recognized widely. It is contemplated that the availability of the protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (1987) Proc. Natl. Acad. Sci. USA 84: 7109-7113. Urist et al. (1984) Proc. Soc. Exp. Biol. Med. 173: 194-199 disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

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Urist et al. (1984) Proc. Natl. Acad. Sci. USA 81:  
371-375 disclose a bovine bone morphogenetic protein  
extract having the properties of an acidic polypeptide  
and a molecular weight of approximately 18 kD. The  
05 authors reported that the protein was present in a  
fraction separated by hydroxyapatite chromatography,  
and that it induced bone formation in mouse hindquarter  
muscle and bone regeneration in trephine defects in rat  
and dog skulls. Their method of obtaining the extract  
10 from bone results in ill-defined and impure  
preparations.

European Patent Application Serial No. 148,155,  
published October 7, 1985, purports to disclose  
osteogenic proteins derived from bovine, porcine, and  
15 human origin. One of the proteins, designated by the  
inventors as a P3 protein having a molecular weight of  
22-24 kD, is said to have been purified to an  
essentially homogeneous state. This material is  
reported to induce bone formation when implanted into  
20 animals.

International Application No. PCT/087/01537,  
published January 14, 1988, discloses an impure  
fraction from bovine bone which has bone induction  
qualities. The named applicants also disclose putative  
25 "bone inductive factors" produced by recombinant DNA  
techniques. Four DNA sequences were retrieved from  
human or bovine genomic or cDNA libraries and expressed  
in recombinant host cells. While the applicants stated  
that the expressed proteins may be bone morphogenic  
30 proteins, bone induction was not demonstrated,  
suggesting that the recombinant proteins are not  
osteogenic. The same group reported subsequently

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(Science 242:1528, Dec, 1988) that three of the four factors induce cartilage formation, and postulate that bone formation activity "is due to a mixture of regulatory molecules" and that "bone formation is most  
05 likely controlled ... by the interaction of these molecules." Again, no bone induction was attributed to the products of expression of the cDNAs. See also Urist et al., EP0,212,474 entitled Bone Morphogenic Agents.

10 Wang et al. (1988) Proc. Nat. Acad. Sci. USA 85: 9484-9488 discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a  
15 molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been  
20 determined.

Wang et al. (1990) Proc. Nat. Acad. Sci. USA 87: 2220-2227 describes the expression and partial purification of one of the cDNA sequences described in PCT 87/01537. Consistent cartilage and/or bone  
25 formation with their protein requires a minimum of 600 ng of 50% pure material.

International Application No. PCT/89/04458 published April 19, 1990 (Int. Pub. No. WO90/003733), describes the purification and analysis of a family of  
30 osteogenic factors called "P3 OF 31-34". The protein family contains at least four proteins, which are characterized by peptide fragment sequences. The

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impure mixture P3 OF 31-34 is assayed for osteogenic activity. The activity of the individual proteins is neither assessed nor discussed.

It is an object of this invention to provide novel polypeptide chains useful as subunits of dimeric osteogenic proteins capable of endochondral bone formation in allogenic and xenogenic implants in mammals, including humans. Another object is to provide genes encoding these polypeptide chains and to provide methods for the production of osteogenic proteins comprising these polypeptide chains using recombinant DNA techniques, as well as to provide antibodies capable of binding specifically to epitopes on these proteins.

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

#### Summary of the Invention

This invention provides novel polypeptide chains useful as either one or both subunits of dimeric osteogenic proteins which, when implanted in a mammalian body in association with a matrix, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation.

A key to these developments was the elucidation of amino acid sequence and structure data of native bovine osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from bovine bone having a half-

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maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The availability of the material enabled the inventors to elucidate key structural details of the protein necessary to achieve bone  
05 formation. Knowledge of the protein's amino acid sequence and other structural features enabled the identification and cloning of native genes in the human genome.

Consensus DNA sequences based on partial sequence  
10 data and observed homologies with regulatory proteins disclosed in the literature were used as probes for extracting genes encoding osteogenic protein from human genomic and cDNA libraries. One of the consensus sequences was used to isolate a previously unidentified  
15 gene which, when expressed, encoded a protein comprising a region capable of inducing endochondral bone formation when properly modified, incorporated in a suitable matrix, and implanted as disclosed herein. The gene, called "hOP1" or "OP-1", is described in  
20 greater detail in U.S. 660,162, filed 27-SEP-91 the disclosure of which is herein incorporated by reference.

Fragments of the hOP1 DNA sequence subsequently were used to probe a mouse embryo cDNA library in  
25 search of additional genes encoding osteogenic proteins. This process isolated a heretofore unidentified DNA sequence which encodes a polypeptide chain referred to herein as mOP1 protein. Mouse OP1 (mOP-1) protein shares significant amino acid sequence  
30 homology with human hOP1 protein, particularly in the region encoding the mature protein. Based on detailed structural and physical analyses of hOP1 protein and the high degree of amino acid sequence homology between



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the hOP1 and mOP-1 proteins, homodimers of mOP1 proteins and heterodimers comprising mOP1 protein are believed to be capable of inducing endochondral bone formation, when the protein is dispersed in a suitable matrix, and implanted as disclosed herein.

The sequence of what is believed to be the mature form of the murine protein, designated herein mOP1-S, is (residues 292-430 of Seq. ID No. 1). The amino acid sequence of the full length protein, mOP1-PP (the "prepro" form, see infra), and the cDNA sequence encoding it are set forth in Seq. ID No. 1.

The invention provides recombinant dimeric proteins, and osteogenic devices comprising these proteins, wherein the subunits of the osteogenic dimers comprise an amino acid sequence described by Seq. ID No. 1, including allelic and biosynthetically mutated variants thereof.

Mouse OP1 can be expressed from intact or truncated cDNA or from synthetic DNAs in procaryotic or eucaryotic host cells, and then purified, cleaved, refolded, dimerized, and implanted in experimental animals. Currently preferred host cells include E. coli, or mammalian cells, such as CHO, COS or BSC cells. The osteogenic protein of the invention may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native or biosynthetic protein, produced by expression of recombinant DNA in host cells.

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Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different species which encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of inducing bone formation in mammals including humans.

In view of this disclosure, and using standard immunology techniques well known in the art, those skilled in the art also may raise polyclonal or monoclonal antibodies against all or part of the polypeptide chains described herein. Useful protocols for antibody production may be found, for example, in Molecular Cloning-A Laboratory Manual (Sambrook et al., eds.) Cold Spring Harbor Press 2nd ed. 1989). See Book 3, Section 18.

The osteogenic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles of porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850 mm, preferably 150mm - 420mm. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently

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preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and specially treated particulate, protein extracted, demineralized, xenogenic bone. Optionally, such

05 xenogenic bone powder matrices also may be treated with proteases such as trypsin and/or fibril modifying agents to increase the intraparticle intrusion volume and surface area. Useful agents include solvents such as dichloromethane, trichloroacetic acid, acetonitrile

10 and acids such as trifluoroacetic acid and hydrogen fluoride. Alternatively, the matrix may be treated with a hot aqueous medium having a temperature within the range of about 37°C to 75°C, including heated acidic aqueous medium. Other potentially useful matrix

15 materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

The osteogenic proteins and implantable osteogenic

20 devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1: 959-963). The

25 devices may be used to induce local endochondral bone formation in non-union fractures as demonstrated in animal tests, and in other clinical applications including dental and periodontal applications where bone formation is required. Another potential clinical

30 application is in cartilage repair, for example, in the treatment of osteoarthritis.

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Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following  
05 description, when read together with the accompanying drawings, in which:

FIGURE 1 compares the amino acid sequences of the mature hOP1 and mOP1 polypeptide chains: OP1-18 and mOP1-S.

10 Description

Purification protocols first were developed which enabled isolation of the osteogenic protein present in crude protein extracts from mammalian bone. (See PCT US 89/01453, and U.S. Serial No. 179,406 filed April 8,  
15 1988, now U.S. Patent No. 4,968,950). The development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (bOP). bOP was characterized significantly; its ability to induce  
20 cartilage and ultimately endochondral bone growth in cat, rabbit, and rat were demonstrated and studied; it was shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone  
25 extracts. This dose dependent and highly specific activity was present whether or not the protein was glycosylated (see (1990) J. Biol. Chem. 265: 13198-13205). Sequence data obtained from the bovine materials suggested probe designs which were used to  
30 isolate human genes. The OP human counterpart proteins have now been expressed and extensively characterized.

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These discoveries enabled preparation of DNAs encoding totally novel, non-native protein constructs which individually as homodimers and combined with other species as heterodimers are capable of producing true endochondral bone (see PCT WO 89/09788, published 19-OCT-89 and US Serial No. 315,342, filed 23-FEB-89, now U.S. Patent No. 5,011,691.) They also permitted expression of the natural material, truncated forms, muteins, analogs, fusion proteins, and various other variants and constructs, from cDNAs and genomic DNAs retrieved from natural sources or from synthetic DNA produced using the techniques disclosed herein and using automated, commercially available equipment. The DNAs may be expressed using well established molecular biology and recombinant DNA techniques in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary, to produce biologically active protein.

One of the DNA sequences isolated from human genomic and cDNA libraries encoded a previously unidentified gene, referred to herein as hOP1. The protein encoded by the isolated DNA was identified originally by amino acid homology with proteins in the TGF- $\beta$  family. Consensus splice signals were found where amino acid homologies ended, designating exon-intron boundaries. Three exons were combined to obtain a functional TGF- $\beta$  like domain containing seven cysteines. (See, for example, U.S. Patent No. 5,011,691 or Ozkaynak, E. et al., (1990) EMBO. 9: pp. 2085-2093). The DNA also is referred to in related applications as "OP1 and "OP-1".

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In its native form, hOP1 expression yields an immature translation product ("hOP1-PP", where "PP" refers to "prepro form") of about 400 amino acids that subsequently is processed to yield a mature sequence of 139 amino acids ("OP1-18"). The active region (functional domain) of the protein comprises the C-terminal 97 amino acids of the hOP1 sequence, "OPS", which includes a conserved six cysteine skeleton. A longer active sequence is OP7, comprising the C-terminal 102 amino acids, and which includes a conserved seven cysteine skeleton.

The full length cDNA sequence for hOP1, and its encoded "prepro" form hOP1-PP, which includes an N-terminal signal peptide sequence, are disclosed in Seq. ID No. 3 (residues 1-431). The mature form of hOP1 protein expressed in mammalian cells, designated herein OP1-18, is indicated by residues 293-431 of Seq. ID No. 3.

cDNA sequences encoding the "prepro" form, of the protein and the mature form, as well as various truncated forms of the gene, and fused genes, have been expressed in E. coli (see, for example, U.S. Serial No. 422, 699) and numerous mammalian cells (See, for example, PCT WO 91/05802, published 2-MAY-91, and all have been shown to have osteogenic activity when implanted in a mammal in association with a suitable matrix.

Given the foregoing amino acid and DNA sequence information, various nucleic acids (RNAs and DNAs) can be constructed which encode at least the active region of an OP1 protein (e.g., OPS or OP7, amino acid residues 335-431 or 330-431, respectively, of Seq. ID

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No. 3) and various analogs thereof, as well as fusion proteins, truncated forms of the mature proteins, and similar constructs. Moreover, DNA hybridization probes can be constructed from fragments of the hOP1 DNA or  
05 designed de novo based on the hOP1 DNA or amino acid sequence. These probes then can be used to screen different genomic and cDNA libraries to identify additional osteogenic proteins.

The DNAs can be produced by those skilled in the  
10 art using well known DNA manipulation techniques involving genomic and cDNA isolation, construction of synthetic DNA from synthesized oligonucleotides, and cassette mutagenesis techniques. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA  
15 Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer. The DNA may then be electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks  
20 which may also be purified by PAGE.

DNAs for use as hybridization probes may be labelled (e.g., as with a radioisotope, by nick translation) and used to identify clones in a given library containing DNA to which the probe hybridizes,  
25 following techniques well known in the art. The libraries may be obtained commercially or they may be constructed de novo using conventional molecular biology techniques. Further information on DNA library construction and hybridization techniques can be found  
30 in numerous texts known to those skilled in the art. See, for example, F.M. Ausubel, ed., Current Protocols in Molecular Biology-Vol. 1, (1989). In particular,

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see unit 5, "Construction of Recombinant DNA Libraries" and Unit 6, "Screening of Recombinant Libraries."

Appropriately identified clones then can be sequenced using any of a number of techniques well known in the art. A DNA fragment containing the sequence of interest then can be subcloned into an expression vector and transfected into an appropriate host cell for protein expression and further characterization. The host may be a procaryotic or eucaryotic cell since the former's inability to glycosylate protein will not destroy the protein's osteogenic activity. Useful host cells include E. coli, Saccharomyces, the insect/baculovirus cell system, myeloma cells, and various mammalian cells. The vector additionally may encode various sequences to promote correct expression of the recombinant protein, including transcription promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence encoding the gene of interest also may be manipulated to remove potentially inhibiting sequences or to minimize unwanted secondary structure formation. The recombinant osteogenic protein also may be expressed as a fusion protein. After being translated, the protein may be purified from the cells themselves or recovered from the culture medium. All biologically active protein forms comprise dimeric species joined by disulfide bonds or otherwise associated, produced by oxidizing and refolding one or more of the various recombinant proteins within an appropriate eucaryotic cell or in vitro after expression of individual subunits. A detailed description of osteogenic protein



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expressed from recombinant DNA in E. coli is disclosed in U.S. Serial No. 660,162, the disclosure of which has been incorporated by reference, supra. A detailed description of osteogenic protein expressed from  
05 recombinant DNA in numerous different mammalian cells is disclosed in PCT WO 91/05802.

#### Exemplification

In an effort to identify additional DNA sequences encoding osteogenic proteins, a hybridization probe  
10 specific to the C-terminus of the DNA of mature hOP1 was prepared using a StuI-EcoRI digest fragment of hOP1 (base pairs 1034-1354 in Seq. ID No. 3), and labelled with  $^{32}\text{P}$  by nick translation, as described in the art. The C-terminus of the protein encodes a key functional  
15 domain e.g., the "active region" for osteogenic activity. The C-terminus also is the region of the protein whose amino acid sequence shares specific amino acid sequence homology with particular proteins in the TGF- $\beta$  super-family of regulatory proteins and which  
20 includes the conserved cysteine skeleton.

Approximately  $7 \times 10^5$  phages of an oligo (dT) primed 17.5 days p.c. mouse embryo 5' stretch cDNA (gt10) library (Clontech, Inc., Palo Alto, CA) was screened with the labelled probe. The screen was  
25 performed using the following stringent hybridization conditions: 40% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, at 37°C overnight, and washing in 0.1 x SSPE, 0.1% SDS, at 50°C.

Five recombinant phages were purified over three  
30 rounds of screening. Phage DNA was prepared from all five phages, subjected to an EcoRI digest, subcloned

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into the EcoR1 site of common pUC-type plasmid modified to allow single strand sequencing, and sequenced using means well known in the art.

Two different DNA sequences were identified by this procedure. One DNA, referred to herein as mOP2, is described in detail in copending USSN 599,543, filed 18-Oct-90. A second DNA, referred to herein as mOP1, is described below.

The cDNA and encoded amino acid sequence for the full length mOP1 protein is depicted in Seq. ID No. 1. The full-length form of the protein is referred to as the prepro form of mOP-1 ("mOP1-PP"), and includes a signal peptide sequence at its N-terminus. The amino acid sequence Ser-Ala-Leu-Ala-Asp (amino acid residues 26-30 in Seq. ID No. 1) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Ser-Ile-Arg-Ser (amino acid residue nos. 288-292 in Seq. ID No. 1) is believed to constitute the cleavage site that produces the mature form of the protein, herein referred to as "mOP1-S" and described by amino acid residues 292-430 of Seq. ID No. 1. The region of the mOP1 amino acid sequence corresponding to the conserved six cysteine skeleton is described by residues 334-430 of Seq. ID No. 1. The region corresponding to the conserved seven cystein skeleton is described by residues 329-430 of Seq. ID No. 1.

Figure 1 compares the amino acid sequence homology of the mature hOP1 and mOP1 proteins (OP1-18 and mOP1-S). Amino acid identity is indicated by three

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dots (...). As can be seen in Figure 1, the mature form of mOP1, mOP1-S shows significant sequence homology with OP-1-18 (98%), differing at only three positions in this region. Like OP-1-18, mOP1-S has a seven cysteine functional domain (residues 38-139 of Fig. 1). The prepro form of the mOP1 protein shares substantially less amino acid sequence homology with that of OP1-PP. The high degree of homology of the mature domains is not surprising as the amino acid sequences of the mature forms of TGF- $\beta$ -like proteins generally also have been found to be highly conserved across different animal species (e.g., compare Vgr and Vgl, two related genes from mouse and Xenopus, respectively, see U.S. Pat. No. 5,011,691). The high degree of amino acid sequence homology exhibited between the mature forms of the two animal species of OP1 proteins identified suggests that the mOP-1 protein will purify essentially as the human OP1 protein does, or with only minor modifications of the protocols disclosed for human OP1 protein. Similarly, purified mOP1-S is predicted to have an apparent molecular weight of about 36 kD as a glycosylated oxidized homodimer, and about 18 kD as a reduced single subunit, as determined by comparison with molecular weight standards on an SDS-polyacrylamide electrophoresis gel. There appear to be three potential N glycosylation sites in the mature mOP1 protein. The unglycosylated homodimer (e.g., one expressed from E. coli) is predicted to have a molecular weight of about 27 kD.

30

## MATRIX PREPARATION

## A. General Consideration of Matrix Properties

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The currently preferred carrier material is a xenogenic bone-derived particulate matrix treated as disclosed herein. This carrier may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., hydroxylapatite (HAP), collagen, tricalcium phosphate or polylactic acid, polyglycolic acid and various copolymers thereof.)

Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75  $\mu\text{m}$  and 420  $\mu\text{m}$  elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate OP onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

The sequential cellular reactions in the interface of the bone matrix/osteogenic protein implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response

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during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible in vivo and preferably biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

Matrix geometry, particle size, the presence of surface charge, and the degree of both intra-and-inter-particle porosity are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogenic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particle and the dispersed osteogenic protein.

The preferred matrix material, prepared from xenogenic bone and treated as disclosed herein, produces an implantable material useful in a variety of clinical settings. In addition to its use as a matrix

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for bone formation in various orthopedic, periodontal, and reconstructive procedures, the matrix also may be used as a sustained release carrier, or as a collagenous coating for implants. The matrix may be  
05 shaped as desired in anticipation of surgery or shaped by the physician or technician during surgery. Thus, the material may be used for topical, subcutaneous, intraperitoneal, or intramuscular implants; it may be  
10 shaped to span a nonunion fracture or to fill a bone defect. In bone formation or conduction procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

Various growth factors, hormones, enzymes,  
15 therapeutic compositions, antibiotics, and other body treating agents also may be absorbed onto the carrier material and will be released over time when implanted as the matrix material is slowly absorbed. Thus, various known growth factors such as EGF, PDGF, IGF,  
20 FGF, TGF- $\alpha$ , and TGF- $\beta$  may be released in vivo. The material can be used to release chemotherapeutic agents, insulin, enzymes, or enzyme inhibitors.

## B. Bone-Derived Matrices

### 1. Preparation of Demineralized Bone

25 Demineralized bone matrix, preferably bovine bone matrix, is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595). Bovine diaphyseal bones (age 1-10 days) are obtained from a local slaughterhouse and  
30 used fresh. The bones are stripped of muscle and fat,

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cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by crushing and pulverized in a large mill. Care is taken to  
05 prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size in the range of 70-850  $\mu\text{m}$ , preferably 150-420  $\mu\text{m}$ , and is defatted by two washes of approximately two hours duration with three volumes of chloroform and methanol  
10 (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether yielding defatted bone powder. The defatted bone powder is then demineralized by four successive treatments with 10 volumes of 0.5 N HCl at  
15 4°C for 40 min. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

## 2. Guanidine Extraction

Demineralized bone matrix thus prepared is  
20 extracted with 5 volumes of 4 M guanidine-HCl, 50mM Tris-HCl, pH 7.0 for 16 hr. at 4°C. The suspension is filtered. The insoluble material is collected and used to fabricate the matrix. The material is mostly collagenous in nature. It is devoid of osteogenic or  
25 chondrogenic activity.

## 3. Matrix Treatments

The major component of all bone matrices is Type-I collagen. In addition to collagen, demineralized bone extracted as disclosed above  
30 includes non-collagenous proteins which may account for 5% of its mass. In a xenogenic matrix, these

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noncollagenous components may present themselves as potent antigens, and may constitute immunogenic and/or inhibitory components. These components also may inhibit osteogenesis in allogenic implants by  
05 interfering with the developmental cascade of bone differentiation. It has been discovered that treatment of the matrix particles with a collagen fibril-modifying agent extracts potentially unwanted components from the matrix, and alters the surface  
10 structure of the matrix material. Useful agents include acids, organic solvents or heated aqueous media. Various treatments are described below. A detailed physical analysis of the effect these fibril-modifying agents have on demineralized, guanidine-  
15 extracted bone collagen particles is disclosed in copending U.S. Patent Application Serial No. 483,913, filed February 22, 1990.

After contact with the fibril-modifying agent, the treated matrix is washed to remove any extracted  
20 components, following a form of the procedure set forth below:

1. Suspend in TBS (Tris-buffered saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) or water and  
25 stir at room temperature (RT) for 30 minutes (sufficient time to neutralize the pH);

2. Centrifuge and repeat wash step; and

3. Centrifuge; discard supernatant; water wash residue; and then lyophilize.

30 3.1 Acid Treatments



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### 1. Trifluoroacetic acid.

Trifluoroacetic acid is a strong non-oxidizing acid that is a known swelling agent for proteins, and which modifies collagen fibrils.

05           Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. These particles are extracted with various percentages (1.0% to 100%) of trifluoroacetic acid and water (v/v) at 0°C or room temperature for 1-2  
10 hours with constant stirring. The treated matrix is filtered, lyophilized, or washed with water/salt and then lyophilized.

### 2. Hydrogen Fluoride.

          Like trifluoroacetic acid, hydrogen fluoride  
15 is a strong acid and swelling agent, and also is capable of altering intraparticle surface structure. Hydrogen fluoride is also a known deglycosylating agent. As such, HF may function to increase the osteogenic activity of these matrices by removing the  
20 antigenic carbohydrate content of any glycoproteins still associated with the matrix after guanidine extraction.

          Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are  
25 collected. The sample is dried in vacuo over  $P_2O_5$ , transferred to the reaction vessel and exposed to anhydrous hydrogen fluoride (10-20 ml/g of matrix) by distillation onto the sample at -70°C. The vessel is allowed to warm to 0°C and the reaction mixture is

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stirred at this temperature for 120 minutes. After evaporation of the hydrogen fluoride in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid. Extent of  
05 deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with hydrogen fluoride, after washing the samples appropriately to remove non-covalently bound carbohydrates. SDS-extracted protein from HF-treated  
10 material is negative for carbohydrate as determined by Con A blotting.

The deglycosylated bone matrix is next washed twice in TBS (Tris-buffered saline) or UTBS, water-washed, and then lyophilized.

15 Other acid treatments are envisioned in addition to HF and TFA. TFA is a currently preferred acidifying reagent in these treatments because of its volatility. However, it is understood that other, potentially less caustic acids may be used, such as  
20 acetic or formic acid.

### 3.2 Solvent Treatment

#### 1. Dichloromethane.

Dichloromethane (DCM) is an organic solvent capable of denaturing proteins without affecting their  
25 primary structure. This swelling agent is a common reagent in automated peptide synthesis, and is used in washing steps to remove components.

Bovine bone residue, prepared as described above, is sieved, and particles of the appropriate size

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are incubated in 100% DCM or, preferably, 99.9% DCM/0.1% TFA. The matrix is incubated with the swelling agent for one or two hours at 0°C or at room temperature. Alternatively, the matrix is treated with  
05 the agent at least three times with short washes (20 minutes each) with no incubation.

## 2. Acetonitrile.

Acetonitrile (ACN) is an organic solvent, capable of denaturing proteins without affecting their  
10 primary structure. It is a common reagent used in high-performance liquid chromatography, and is used to elute proteins from silica-based columns by perturbing hydrophobic interactions.

Bovine bone residue particles of the  
15 appropriate size, prepared as described above, are treated with 100% ACN (1.0 g/30 ml) or, preferably, 99.9% ACN/0.1% TFA at room temperature for 1-2 hours with constant stirring. The treated matrix is then water-washed, or washed with urea buffer, or 4 M NaCl  
20 and lyophilized. Alternatively, the ACN or ACN/TFA treated matrix may be lyophilized without wash.

## 3. Isopropanol.

Isopropanol is also an organic solvent capable of denaturing proteins without affecting their primary  
25 structure. It is a common reagent used to elute proteins from silica HPLC columns.

Bovine bone residue particles of the appropriate size prepared as described above are treated with 100% isopropanol (1.0 g/30 ml) or,

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preferably, in the presence of 0.1% TFA, at room temperature for 1-2 hours with constant stirring. The matrix is then water-washed or washed with urea buffer or 4 M NaCl before being lyophilized.

05                   4.    Chloroform

Chloroform also may be used to increase surface area of bone matrix like the reagents set forth above, either alone or acidified.

Treatment as set forth above is effective to  
10 assure that the material is free of pathogens prior to implantation.

3.3   Heat Treatment

The currently most preferred agent is a heated aqueous fibril-modifying medium such as water, to  
15 increase the matrix particle surface area and porosity. The currently most preferred aqueous medium is an acidic aqueous medium having a pH of less than about 4.5, e.g., within the range of pH 2 - pH 4. which may help to "swell" the collagen before heating. 0.1%  
20 acetic acid, which has a pH of about 3, currently is preferred. 0.1 M acetic acid also may be used.

Various amounts of delipidated, demineralized guanidine-extracted bone collagen are heated in the aqueous medium (1g matrix/30ml aqueous medium) under  
25 constant stirring in a water jacketed glass flask, and maintained at a given temperature for a predetermined period of time. Preferred treatment times are about one hour, although exposure times of between about 0.5 to two hours appear acceptable. The temperature

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employed is held constant at a temperature within the range of about 37°C to 75°C. The currently preferred heat treatment temperature is within the range of 45°C to 60°C.

05 After the heat treatment, the matrix is filtered, washed, lyophilized and used for implant. Where an acidic aqueous medium is used, the matrix also is preferably neutralized prior to washing and lyophilization. A currently preferred neutralization  
10 buffer is a 200mM sodium phosphate buffer, pH 7.0. To neutralize the matrix, the matrix preferably first is allowed to cool following thermal treatment, the acidic aqueous medium (e.g., 0.1% acetic acid) then is removed and replaced with the neutralization buffer and the  
15 matrix agitated for about 30 minutes. The neutralization buffer then may be removed and the matrix washed and lyophilized (see infra).

The matrix also may be treated to remove contaminating heavy metals, such as by exposing the  
20 matrix to a metal ion chelator. For example, following thermal treatment with 0.1% acetic acid, the matrix may be neutralized in a neutralization buffer containing EDTA (sodium ethylenediaminetetraacetic acid), e.g., 200 mM sodium phosphate, 5mM EDTA, pH 7.0. 5 mM EDTA  
25 provides about a 100-fold molar excess of chelator to residual heavy metals present in the most contaminated matrix tested to date. Subsequent washing of the matrix following neutralization appears to remove the bulk of the EDTA. EDTA treatment of matrix particles  
30 reduces the residual heavy metal content of all metals tested (Sb, As, Be, Cd, Cr, Cu, Co, Pb, Hg, Ni, Se, Ag, Zn, Tl) to less than about 1 ppm. Bioassays with EDTA-

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treated matrices indicate that treatment with the metal ion chelator does not inhibit bone inducing activity.

The collagen matrix materials preferably take the form of a fine powder, insoluble in water, comprising nonadherent particles. It may be used simply by packing into the volume where new bone growth or sustained release is desired, held in place by surrounding tissue. Alternatively, the powder may be encapsulated in, e.g., a gelatin or polylactic acid coating, which is adsorbed readily by the body. The powder may be shaped to a volume of given dimensions and held in that shape by interadhering the particles using, for example, soluble, species-biocompatible collagen. The material may also be produced in sheet, rod, bead, or other macroscopic shapes.

#### FABRICATION OF OSTEOGENIC DEVICE

The naturally sourced and recombinant protein as set forth above, and other constructs, can be combined and dispersed in a suitable matrix preparation using any of the methods described below. In general, 50-100 ng of active protein is combined with the inactive carrier matrix (e.g., 25 mg for rat bioassays). Greater amounts may be used for large implants.

##### 1. Ethanol Precipitation

Matrix is added to osteogenic protein dissolved in guanidine-HCl. Samples are vortexed and incubated at a low temperature (e.g., 4°C). Samples are then further vortexed. Cold absolute ethanol (5 volumes) is added to the mixture which is then stirred

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and incubated, preferably for 30 minutes at  $-20^{\circ}\text{C}$ . After centrifugation (microfuge, high speed) the supernatant is discarded. The reconstituted matrix is washed twice with cold concentrated ethanol in water  
05 (85% EtOH) and then lyophilized.

## 2. Acetonitrile Trifluoroacetic Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution is  
10 added to the carrier material. Samples are vigorously vortexed many times and then lyophilized. This method is currently preferred, and has been tested with osteogenic protein at varying concentrations and different levels of purity.

## 15 3. Urea Lyophilization

For those osteogenic proteins that are prepared in urea buffer, the protein is mixed with the matrix material, vortexed many times, and then lyophilized. The lyophilized material may be used "as  
20 is" for implants.

## 4. Buffered Saline Lyophilization

CP1 preparations in physiological saline may also be vortexed with the matrix and lyophilized to produce osteogenically active material.

25 These procedures also can be used to adsorb other active therapeutic drugs, hormones, and various bioactive species to the matrix for sustained release purposes.

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## BIOASSAY

The functioning of the various proteins and devices of this invention can be evaluated with an in vivo bioassay. Studies in rats show the osteogenic effect in an appropriate matrix to be dependent on the dose of osteogenic protein dispersed in the matrix. No activity is observed if the matrix is implanted alone. In vivo bioassays performed in the rat model also have shown that demineralized, guanidine-extracted xenogenic bone matrix materials of the type described in the literature are ineffective as a carrier, fail to induce bone, and produce an inflammatory and immunological response when implanted unless treated as disclosed above. In certain species (e.g., monkey) allogenic matrix materials also apparently are ineffective as carriers. The following sets forth various procedures for preparing osteogenic devices from the proteins and matrix materials prepared as set forth above, and for evaluating their osteogenic utility.

20 A. Rat Model

## 1. Implantation

The bioassay for bone induction as described by Sampath and Reddi ((1983) Proc. Natl. Acad. Sci. USA 80 6591-6595), herein incorporated by reference, may be used to monitor endochondral bone differentiation activity. This assay consists of implanting test samples in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic



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region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The heterotrophic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotrophic sites. As disclosed herein, both allogenic (rat bone matrix) and xenogenic (bovine bone matrix) implants were assayed.

## 2. Cellular Events

Successful implants exhibit a controlled progression through the stages of protein-induced endochondral bone development, including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

## 3. Histological Evaluation

Histological sectioning and staining is preferred to determine the extent of osteogenesis in

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the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8  $\mu$ m sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually sufficient to determine whether the implants contain newly induced bone.

#### 4. Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation quickly after the implants are removed from the rat. Alternatively, the amount of bone formation can be determined by measuring the calcium content of the implant.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 05 (i) APPLICANT:  
OZKAYNAK, ENGIN  
OPPERMANN, HERMANN  
KUBERASAMPATH, THANGAVEL  
RUEGER, DAVID C.
- (ii) TITLE OF INVENTION: OSTEOGENIC DEVICES
- (iii) NUMBER OF SEQUENCES: 4
- 10 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: TESTA, HURWITZ & THIBEAULT  
(B) STREET: 53 STATE STREET  
(C) CITY: BOSTON  
(D) STATE: MASSACHUSETTS  
15 (E) COUNTRY: U.S.A.  
(F) ZIP: 02109
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
10 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: Patent In Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
25 (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: PITCHER, EDMUND R.  
(B) REGISTRATION NUMBER: 27,829  
(C) REFERENCE/DOCKET NUMBER: CRP-001PC5
- 30 (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 617/248-7000  
(B) TELEFAX: 617/248-7100

## (2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1873 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

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(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: MURIDAE  
 (F) TISSUE TYPE: EMBRYO

05 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 104..1393  
 (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"  
 /product= "mOP1-PP"  
 /note= "mOP1 (CDNA)"

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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- 36 -

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	425	430		
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 (A) LENGTH: 430 amino acids  
 (B) TYPE: amino acid  
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- (ix) FEATURE:  
 (D) OTHER INFORMATION: /product= "mOP1-PP"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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- 38 -

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 305 310 315 320  
 05 Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val  
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 10 355 360 365  
 Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe  
 370 375 380  
 Ile Asn Pro Asp Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu  
 385 390 395 400  
 15 Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Asp Leu  
 405 410 415  
 Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His  
 420 425 430

## (2) INFORMATION FOR SEQ ID NO:3:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1822 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 25 (ii) MOLECULE TYPE: cDNA  
 (iii) HYPOTHETICAL: NO  
 (iv) ANTI-SENSE: NO  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: HOMO SAPIENS  
 30 (F) TISSUE TYPE: HIPPOCAMPUS  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 49..1341  
 (C) IDENTIFICATION METHOD: experimental  
 35 (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"  
 /product= "hOP1-PP"  
 /evidence= EXPERIMENTAL



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/standard\_name= "hOP1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

05	GGTGC	GGGCC	CGGAG	CCCCGG	AGCCCCGGTA	GCGCGTAGAG	CCGGCGCG	ATG	CAC	GTG	57						
								Met	His	Val							
								1									
	CGC	TCA	CTG	CGA	GCT	GCG	GCG	CCG	CAC	AGC	TTC	GTG	GCG	CTC	TGG	GCA	105
	Arg	Ser	Leu	Arg	Ala	Ala	Ala	Pro	His	Ser	Phe	Val	Ala	Leu	Trp	Ala	
		5						10					15				
10	CCC	CTG	TTC	CTG	CTG	CGC	TCC	GCC	CTG	GCC	GAC	TTC	AGC	CTG	GAC	AAC	153
	Pro	Leu	Phe	Leu	Leu	Arg	Ser	Ala	Leu	Ala	Asp	Phe	Ser	Leu	Asp	Asn	
	20					25					30					35	
	GAG	GTG	CAC	TCG	AGC	TTC	ATC	CAC	CGG	CGC	CTC	CGC	AGC	CAG	GAG	CGG	201
	Glu	Val	His	Ser	Ser	Phe	Ile	His	Arg	Arg	Leu	Arg	Ser	Gln	Glu	Arg	
					40					45					50		
15	CGG	GAG	ATG	CAG	CGC	GAG	ATC	CTC	TCC	ATT	TTG	GGC	TTG	CCC	CAC	CGC	249
	Arg	Glu	Met	Gln	Arg	Glu	Ile	Leu	Ser	Ile	Leu	Gly	Leu	Pro	His	Arg	
				55				60						65			
	CCG	CGC	CCG	CAC	CTC	CAG	GGC	AAG	CAC	AAC	TCG	GCA	CCC	ATG	TTC	ATG	297
	Pro	Arg	Pro	His	Leu	Gln	ly	Lys	His	Asn	Ser	Ala	Pro	Met	Phe	Met	
20			70					75					80				
	CTG	GAC	CTG	TAC	AAC	GCC	ATG	GCG	GTG	GAG	GAG	GGC	GGC	GGG	CCC	GGC	345
	Leu	Asp	Leu	Tyr	Asn	Ala	Met	Ala	Val	Glu	Glu	Gly	Gly	Gly	Pro	Gly	
		85					90					95					
	GGC	CAG	GGC	TTC	TCC	TAC	CCC	TAC	AAG	GCC	GTC	TTC	AGT	ACC	CAG	GGC	393
25	Gly	Gln	Gly	Phe	Ser	Tyr	Pro	Tyr	Lys	Ala	Val	Phe	Ser	Thr	Gln	Gly	
	100					105					110					115	
	CCC	CCT	CTG	GCC	AGC	CTG	CAA	GAT	AGC	CAT	TTC	CTC	ACC	GAC	GCC	GAC	441
	Pro	Pro	Leu	Ala	Ser	Leu	Gln	Asp	Ser	His	Phe	Leu	Thr	Asp	Ala	Asp	
				120						125					130		
30	ATG	GTC	ATG	AGC	TTC	GTC	AAC	CTC	GTG	GAA	CAT	GAC	AAG	GAA	TTC	TTC	489
	Met	Val	Met	Ser	Phe	Val	Asn	Leu	Val	Glu	His	Asp	Lys	Glu	Phe	Phe	
				135					140					145			
	CAC	CCA	CGC	TAC	CAC	CAT	CGA	GAG	TTC	CGG	TTT	GAT	CTT	TCC	AAG	ATC	537
	His	Pro	Arg	Tyr	His	His	Arg	Glu	Phe	Arg	Phe	Asp	Leu	Ser	Lys	Ile	
35			150					155					160				
	CCA	GAA	GGG	GAA	GCT	GTC	ACG	GCA	GCC	GAA	TTC	CGG	ATC	TAC	AAG	GAC	585
	Pro	Glu	Gly	Glu	Ala	Val	Thr	Ala	Ala	Glu	Phe	Arg	Ile	Tyr	Lys	Asp	
		165					170					175					
	TAC	ATC	CGG	GAA	CGC	TTC	GAC	AAT	GAG	ACG	TTC	CGG	ATC	AGC	GTT	TAT	633

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	Tyr	Ile	Arg	Glu	Arg	Phe	Asp	Asn	Glu	Thr	Phe	Arg	Ile	Ser	Val	Tyr	
	180					185					190					195	
05	CAG	GTG	CTC	CAG	GAG	CAC	TTG	GGC	AGG	GAA	TCG	GAT	CTC	TTC	CTG	CTC	681
	Gln	Val	Leu	Gln	Glu	His	Leu	Gly	Arg	Glu	Ser	Asp	Leu	Phe	Leu	Leu	
				200						205					210		
	GAC	AGC	CGT	ACC	CTC	TGG	GCC	TCG	GAG	GAG	GGC	TGG	CTG	GTG	TTT	GAC	729
	Asp	Ser	Arg	Thr	Leu	Trp	Ala	Ser	Glu	Glu	Gly	Trp	Leu	Val	Phe	Asp	
				215					220					225			
10	ATC	ACA	GCC	ACC	AGC	AAC	CAC	TGG	GTG	GTC	AAT	CCG	CGG	CAC	AAC	CTG	777
	Ile	Thr	Ala	Thr	Ser	Asn	His	Trp	Val	Val	Asn	Pro	Arg	His	Asn	Leu	
			230					235					240				
	GGC	CTG	CAG	CTC	TCG	GTG	GAG	ACG	CTG	GAT	GGG	CAG	AGC	ATC	AAC	CCC	825
	Gly	Leu	Gln	Leu	Ser	Val	Glu	Thr	Leu	Asp	Gly	Gln	Ser	Ile	Asn	Pro	
		245					250					255					
15	AAG	TTG	GCG	GGC	CTG	ATT	GGG	CGG	CAC	GGG	CCC	CAG	AAC	AAG	CAG	CCC	873
	Lys	Leu	Ala	Gly	Leu	Ile	Gly	Arg	His	Gly	Pro	Gln	Asn	Lys	Gln	Pro	
	260					265					270					275	
	TTC	ATG	GTG	GCT	TTC	TTC	AAG	GCC	ACG	GAG	GTC	CAC	TTC	CGC	AGC	ATC	921
20	Phe	Met	Val	Ala	Phe	Phe	Lys	Ala	Thr	Glu	Val	His	Phe	Arg	Ser	Ile	
					280					285					290		
	CGG	TCC	ACG	GGG	AGC	AAA	CAG	CGC	AGC	CAG	AAC	CGC	TCC	AAG	ACG	CCC	969
	Arg	Ser	Thr	Gly	Ser	Lys	Gln	Arg	Ser	Gln	Asn	Arg	Ser	Lys	Thr	Pro	
				295					300					305			
25	AAG	AAC	CAG	GAA	GCC	CTG	CGG	ATG	GCC	AAC	GTG	GCA	GAG	AAC	AGC	AGC	1017
	Lys	Asn	Gln	Glu	Ala	Leu	Arg	Met	Ala	Asn	Val	Ala	Glu	Asn	Ser	Ser	
			310					315					320				
	AGC	GAC	CAG	AGG	CAG	GCC	TGT	AAG	AAG	CAC	GAG	CTG	TAT	GTC	AGC	TTC	1065
	Ser	Asp	Gln	Arg	Gln	Ala	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val	Ser	Phe	
		325					330					335					
30	CGA	GAC	CTG	GGC	TGG	CAG	GAC	TGG	ATC	ATC	GCG	CCT	GAA	GGC	TAC	GCC	1113
	Arg	Asp	Leu	Gly	Trp	Gln	Asp	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala	
	340					345					350					355	
	GCC	TAC	TAC	TGT	GAG	GGG	GAG	TGT	GCC	TTC	CCT	CTG	AAC	TCC	TAC	ATG	1161
35	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala	Phe	Pro	Leu	Asn	Ser	Tyr	Met	
				360						365					370		
	AAC	GCC	ACC	AAC	CAC	GCC	ATC	GTG	CAG	ACG	CTG	GTC	CAC	TTC	ATC	AAC	1209
	Asn	Ala	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu	Val	His	Phe	Ile	Asn	
				375					380					385			
40	CCG	GAA	ACG	GTG	CCC	AAG	CCC	TGC	TGT	GCG	CCC	ACG	CAG	CTC	AAT	GCC	1257
	Pro	Glu	Thr	Val	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln	Leu	Asn	Ala	

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	390	395	400	
	ATC TCC GTC CTC TAC TTC GAT GAC AGC TCC AAC GTC ATC CTG AAG AAA			1305
	Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys			
	405	410	415	
05	TAC AGA AAC ATG GTG GTC CGG GCC TGT GGC TGC CAC TAGCTCCTCC			1351
	Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His			
	420	425	430	
	GAGAATTCAG ACCCTTTGGG GCCAAGTTTT TCTGGATCCT CCATTGCTCG CCTTGGCCAG			1411
	GAACCAGCAG ACCAACTGCC TTTTGTGAGA CCTTCCCCTC CCTATCCCCA ACTTTAAAGG			1471
10	TGTGAGAGTA TTAGGAAACA TGAGCAGCAT ATGGCTTTTG ATCAGTTTTT CAGTGGCAGC			1531
	ATCCAATGAA CAAGATCCTA CAAGCTGTGC AGGCAAAACC TAGCAGGAAA AAAAAACAAC			1591
	GCATAAAGAA AAATGGCCGG GCCAGGTCAT TGGCTGGGAA GTCTCAGCCA TGCACGGACT			1651
	CGTTTCAGG GGTAAATTATG AGCGCCTACC AGCCAGGCCA CCCAGCCGTG GGAGGAAGGG			1711
	GGCGTGGCAA GGGGTGGGCA CATTGGTGTC TGTGCGAAAG GAAAATTGAC CCGGAAGTTC			1771
15	CTGTAATAAA TGTCACAATA AAACGAATGA ATGAAAAAAA AAAAAAAAAA A			1822

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 431 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(D) OTHER INFORMATION: /Product="hOP1-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25	Met His Val Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala
	1 5 10 15
	Leu Trp Ala Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser
	20 25 30
30	Leu Asp Asn Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser
	35 40 45
	Gln Glu Arg Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu
	50 55 60

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Pro His Arg Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro  
 65 70 75 80  
 Met Phe Met Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly  
 85 90 95  
 05 Gly Pro Gly Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser  
 100 105 110  
 Thr Gln Gly Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr  
 115 120 125  
 10 Asp Ala Asp Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys  
 130 135 140  
 Glu Phe Phe His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu  
 145 150 155 160  
 Ser Lys Ile Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile  
 165 170 175  
 15 Tyr Lys Asp Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile  
 180 185 190  
 Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu  
 195 200 205  
 20 Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu  
 210 215 220  
 Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg  
 225 230 235 240  
 His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser  
 245 250 255  
 25 Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn  
 260 265 270  
 Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe  
 275 280 285  
 30 Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser  
 290 295 300  
 Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu  
 305 310 315 320  
 Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr  
 325 330 335  
 35 Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu  
 340 345 350

- 43 -

	Gly	Tyr	Ala	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala	Phe	Pro	Leu	Asn
			355					360					365			
	Ser	Tyr	Met	Asn	Ala	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu	Val	His
		370					375					380				
05	Phe	Ile	Asn	Pro	Glu	Thr	Val	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln
	385					390					395					400
	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe	Asp	Asp	Ser	Ser	Asn	Val	Ile
					405					410					415	
10	Leu	Lys	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg	Ala	Cys	Gly	Cys	His	
			420						425					430		

What is claimed is:

1. A polypeptide chain comprising an amino acid sequence described by residues 334-430 of Seq. ID No. 1.
- 05 2. The polypeptide chain of claim 1 comprising an amino acid sequence described by residues 329-430 of Seq. ID No. 1.
3. The polypeptide chain of claim 2 comprising an amino acid sequence described by residues 292-430 of Seq. ID No. 1.
- 10 4. The polypeptide chain of claim 3 comprising an amino acid sequence described by residues 1-430 of Seq. ID No. 1.
- 15 5. A polypeptide chain useful as a subunit of a dimeric osteogenic protein, said protein being capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix;  
  
said polypeptide chain comprising an amino  
20 acid sequence described by residues 334-430 of Seq. ID No. 1, including allelic variants thereof.
- 25 6. The polypeptide chain of claim 5 wherein said polypeptide chain comprises the amino acid sequence described by residues 292-430 of Seq. ID No. 1, including allelic variants thereof.:

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7. The polypeptide chain of claim 1 or 5 produced by expression of recombinant DNA in a host cell.
- 05 8. The polypeptide chain of claim 7 wherein said host cell is a eucaryotic host cell.
9. The polypeptide chain of claim 8 wherein said eucaryotic host cell is a mammalian cell.
- 10 10. The polypeptide chain of claim 7 wherein said host cell is a procaryotic host cell.
11. The polypeptide chain of claim 10 wherein said procaryotic host cell is E.coli.
12. The polypeptide chain of claim 1 or 5 that is glycosylated.
- 15 13. A nucleic acid encoding the polypeptide chain of claim 1 or 5.
14. An osteogenic protein capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix; said protein comprising a dimeric species having  
20 two oxidized subunits, the amino acid sequence of each said subunit comprising the amino acid sequence described by residues 334-430 of Seq. ID No.1, including allelic variants thereof.
- 25 15. The osteogenic protein of claim 14 wherein said amino acid sequence comprises the sequence described by residues 292-430 of Seq. ID No. 1, including allelic variants thereof.

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16. An antibody capable of binding to an epitope  
on a protein comprising the amino acid  
sequence described by residues 334-430 of Seq.  
ID No. 1, including allelic variants thereof.



1/3

hOP-1	Ser	Thr	Gly	Ser	Lys	Gln	Arg	Ser	Gln
mOP-1	...	...	...	Gly	...	...	...		
	1				5				

hOP-1	Asn	Arg	Ser	Lys	Thr	Pro	Lys	Asn	Gln
mOP-1	...	...	...	...	...	...	...	...	...
	10					15			

hOP-1	Glu	Ala	Leu	Arg	Met	Ala	Asn	Val	Ala
mOP-1	...	...	...	..	...	...	Ser	...	...
		20					25		

hOP-1	Glu	Asn	Ser	Ser	Ser	Asp	Gln	Arg	Gln
mOP-1	...	...	...	...	...	...	...	...	...
			30					35	

hOP-1	Ala	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val
mOP-1	...	...	...	...	...	...	...	...	...
				40					45

hOP-1	Ser	Phe	Arg	Asp	Leu	Gly	Trp	Gln	Asp
mOP-1	...	...	...	...	...	...	...	...	...
					50				

hOP-1	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala
mOP-1	...	...	...	...	...	...	...	...	...
	55					60			

FIG. 1.1

2/3

hOP-1	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
mOP-1	...	...	...	...	...	...	...	...	...

65

70

hOP-1	Phe	Pro	Leu	Asn	Ser	Tyr	Met	Asn	Ala
mOP-1	...	...	...	...	...	...	...	...	...

75

80

hOP-1	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu
mOP-1	...	...	...	...	...	...	...	...	...

85

90

hOP-1	Val	His	Phe	Ile	Asn	Pro	Glu	Thr	Val
mOP-1	...	...	...	...	...	...	Asp	...	...

95

hOP-1	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln
mOP-1	...	...	...	...	...	...	...	...	...

100

105

hOP-1	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe
mOP-1	...	...	...	...	...	...	...	...	...

110

115

hOP-1	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys
mOP-1	...	...	...	...	...	...	Asp	...	...

120

125

FIG. 1.2

*3/3*

hOP-1	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg
mOP-1	...	...	...	...	...	...	...	...
				130				135

hOP-1	Ala	Cys	Gly	Cys	His
mOP-1	...	...	...	...	...

**FIG. 1.3**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/07654

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>5</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 15/06, C 12 N 15/12, A 61 K 37/02														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">IPC5</td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">C 07 K; C 12 N; A 61 K</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	IPC5	C 07 K; C 12 N; A 61 K								
Classification System	Classification Symbols													
IPC5	C 07 K; C 12 N; A 61 K													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 60%; padding: 2px;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%; padding: 2px;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="padding: 5px; vertical-align: top;">P,X</td> <td style="padding: 5px;">EP, A2, 0416578 (TAKEDA CHEMICAL INDUSTRIES, LTD. ET AL) 13 March 1991, see e.g. fig. 2, fig. 4-3 <div style="text-align: center;">---</div></td> <td style="padding: 5px; vertical-align: top;">1-3,5-16</td> </tr> <tr> <td style="padding: 5px; vertical-align: top;">P,X</td> <td style="padding: 5px;">Proc. Natl. Acad. Sci. USA, vol. 87, December 1990, A.J. Celeste et al: "Identification of transforming growth factor Beta family members present in bone-inductive protein purified from bovine bone", see page 9843 - page 9847 see fig.1 <div style="text-align: center;">---</div></td> <td style="padding: 5px; vertical-align: top;">1-16</td> </tr> <tr> <td style="padding: 5px; vertical-align: top;">X</td> <td style="padding: 5px;">WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see especially table V <div style="text-align: center;">---</div></td> <td style="padding: 5px; vertical-align: top;">1-16</td> </tr> </tbody> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	P,X	EP, A2, 0416578 (TAKEDA CHEMICAL INDUSTRIES, LTD. ET AL) 13 March 1991, see e.g. fig. 2, fig. 4-3 <div style="text-align: center;">---</div>	1-3,5-16	P,X	Proc. Natl. Acad. Sci. USA, vol. 87, December 1990, A.J. Celeste et al: "Identification of transforming growth factor Beta family members present in bone-inductive protein purified from bovine bone", see page 9843 - page 9847 see fig.1 <div style="text-align: center;">---</div>	1-16	X	WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see especially table V <div style="text-align: center;">---</div>	1-16
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X	WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see especially table V <div style="text-align: center;">---</div>	1-16												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <b>20th February 1992</b> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <b>11. 03. 92</b> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority  <b>EUROPEAN PATENT OFFICE</b> </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer  </td> </tr> </table>			Date of the Actual Completion of the International Search <b>20th February 1992</b>	Date of Mailing of this International Search Report <b>11. 03. 92</b>	International Searching Authority <b>EUROPEAN PATENT OFFICE</b>	Signature of Authorized Officer 								
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International Searching Authority <b>EUROPEAN PATENT OFFICE</b>	Signature of Authorized Officer 													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 9003733 (INTERNATIONAL GENETIC ENGINEERING, INC.) 19 April 1990, see the whole document  --	1-16
X	WO, A1, 8909787 (CREATIVE BIOMOLECULES, INC.) 19 October 1989, see especially claim 23  --	1-2,5,7-14,16
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ANNEX TO THE INTERNATIONAL SEARCH REPORT  
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